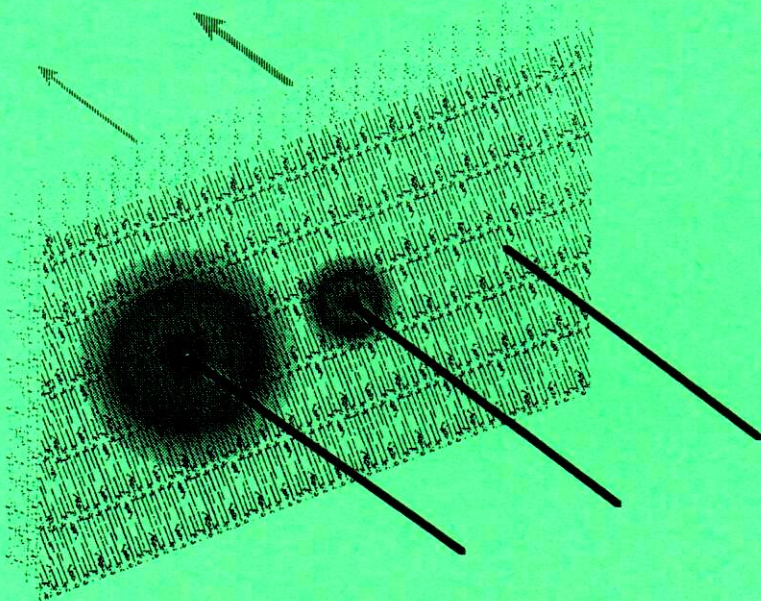


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RECONSTITUTION OF LIPOSOMES INSIDE THE INTERCELLULAR LIPID DOMAIN OF THE STRATUM CORNEUM BY DILUTION OF PHOSPHOLIPID/OCTYL GLUCOSIDE MICELLAR SOLUTIONS

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The use of liposomes in dermatology has become progressively popular in recent decades. However, a controversy exists on the mechanisms of the interaction of these vesicles with the skin. Thus, there is not consensus that lipid vesicles dermally applied really penetrate into skin and on the possible mechanisms of such penetration.

In previous works we have studied the effect of liposomes¹ and different solubilizing agents² on the stratum corneum tissue (SC), as well as the ability of SC lipids to form liposomes³. Taking into account that the structure of liposomes is similar to the layered structure of the SC intercellular lipids, it is reasonable to consider the possibility of application of liposomes as protective agents of this tissue. As a consequence, in this work we sought to evaluate the protective effect caused by PC liposomes on the SC structure against the action of the non-ionic surfactant octyl glucoside (OG). In addition, the ability of these vesicles to be reconstituted inside the SC tissue was examined. These studies were carried out *in vitro* using the technique of high-resolution low-temperature scanning electron microscopy.

Treatment of the SC tissue with OG led to breakage of the corneocyte envelopes and to the diffusion of the protein material from the corneocyte into the intercellular domain. The interaction of this protein material with the intercellular lipids caused a certain alteration in the lipid structure that resulted in the formation of "rough structures"⁴. Previous incubation of SC with PC liposomes drastically reduced these alterations, this protection being associated with incorporation of about 10% of PC to the SC. In addition, in these samples, the presence of intact PC vesicles in the intercellular lipid domain was detected after OG treatment and subsequent washing. This, and the fact that the OG/PC molar ratio incorporated in the SC corresponded to that at which the micelle-lamella transition occurred on dilution demonstrated the ability of PC liposomes to be reconstituted inside the SC. Hence, the presence of PC vesicles inside the SC could be explained by the incorporation of an OG/PC micellar system and the subsequent reconstitution in PC vesicles by continuous dilution during washing.

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RECONSTITUTION OF LIPOSOMES INSIDE THE INTERCELLULAR LIPID DOMAIN OF THE STRATUM CORNEUM BY DILUTION OF PHOSPHOLIPID/OCTYL GLUCOSIDE MICELLAR SOLUTIONS

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INTRODUCTION

A number of studies have been devoted about the mechanisms of the interaction of liposomes with skin. In this sense, various authors recently have claimed the advantages of topical application of phospholipid liposomes [1,2], however, there is not consensus in that lipid vesicles dermally applied really penetrate into the skin and which would be the cause of such possible penetration. Thus, whereas some authors claimed that liposomes are not able to penetrate deeper than 90 [3,4], other reported various effects of these vesicles on the epidermis [5,6].

OBJECTIVE: Use of high-resolution low-temperature scanning electron microscopy technique (HRLTEM) to evaluate the ability of PC liposomes to protect pig SC against the action of the surfactant octyl glucoside and to be reconstituted inside of this tissue.

METHODS

PC liposomes: Preparation following a method previously reported [7].
SC native: Separation and isolation based on a method previously described [10,11].
SC treated with OG: Treatment of SC native with 20 mM OG by sonication at 25°C for 15 min and incubation at the same temperature for 18 h in N₂ atmosphere [12].
SC incubated with liposomes: Incubation with PC liposomes 15 mM at 25°C for 18 h, separation from the liposome solution, treatment with 20 mM OG and washing with buffer during 30 min.

TECHNIQUES

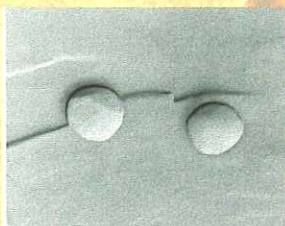
Thin layer chromatography flame ionization detection (TLC-FID) was used to analyze the amounts of PC and OG incorporated in the SC after incubation with liposomes or surfactant treatment respectively [11].

Dynamic light-scattering (DLS) technique was used to determine the hydrodynamic diameter (HD) of PC liposomes and OG/PC mixed micelles [8].

Freeze-fracture electron microscopy (FFEM) study of PC liposomes and reconstituted vesicles by dilution was done according to the procedure described by Egelhaaf et al. [13]. The replicas were examined in a Philips EM 301 electron microscopy at 80 kv.

High-Resolution Low-Temperature Scanning Electron Microscopy (HRLTEM) Cylindrical pieces of each sample were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, cryoprotected with 30% (v/v) glycerol and mounted on a 3 mm aluminium holder. Thereafter, the SC samples were frozen by plunging into liquid propane and fractured in a Balzers BAF 300 freeze-etching device (Bal-Tec., Liechtenstein) at 10⁻⁷ mbar and a temperature of -110°C. After etching for 2 minutes, the fracture plane was coated with 2 nm Pt/C and 5-7 nm C. Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900. In low field emission scanning electron microscope equipped with a highly sensitive anastar YAG-detector for back scattered electrons [9]. Specimens were investigated at -110°C. The beam current was 1-3x10⁻¹¹ A as measured with a Faraday cage. The primary accelerating voltage was 10 kV. Images were obtained with the back scattered electron signal and recorded digitally with a Gatan Digicon 688 connected to an Apple Quadra 950.

RESULTS AND DISCUSSION



FFEM micrograph corresponding to two PC vesicles showing diameters of about 180 nm, in agreement with the DLS results

NATIVE STRATUM CORNEUM



This picture depicts corneocytes (black arrow) characterized by the particular pattern of keratine filling these structures and by the absence of cell organelles. In the intercellular space the fracture plane goes along the lamellae of the multilayered lipid organization resulting on the very smooth and relatively flat surface of the SC lipids (black arrowheads). Occasionally the bilayers were fractured straight across, resulting in sharp edges (white arrows) indicating the multilamellar structure of the intercellular lipids.

STRATUM CORNEUM TREATED WITH OG



The corneocyte showed a more irregular shape than that observed in the native tissue (Fig 2). This perturbation was attributed to a partial breaking of the corneocyte envelope (white open arrow) that induced a release of the protein material into the intercellular lipid region. The presence of flat surfaces corresponding to the fracture along lipid lamellae (black arrowhead) and sharp steps corresponding to the fractures across the lipid lamellae (white arrow) indicates that the lamellar structure remained preserved after the OG treatment, despite the strong effect caused by OG on the protein structure. The main alteration of this lipid region was the formation of a wide area of new rough structure (white arrowhead). This structure, (not observed in the native SC, Fig 2), is very similar to those reported by Hofland et al. and Van Hal et al. [14,15], who associated their formation to the disruption of the lipid lamellar structure. We attribute the formation of this 'rough structure' to the interaction of the protein material liberated from the corneocytes (when the corneocyte envelope was damaged) and the lipids of the intercellular spaces, with the subsequent disorder in their lamellar structure.

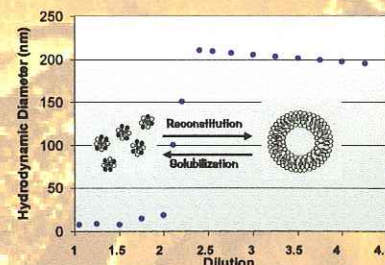
SC INCUBATED WITH LIPOSOMES AND TREATED WITH OG



Figure 4 depicts three SC samples previously incubated with PC liposomes (PC conc 15 mM) and immediately treated with OG. The lipid matrix exhibited the structure of multiple layers (white arrows), indicating the preservation of these structures as occurred in the SC treated with OG without previous incubation (Fig 3). However, the rough structures observed in the SC treated with OG were not observed in any micrograph of this Figure. Hence, the incubation with PC liposomes protected the SC against OG, being this protection associated to the incorporation of about 10% of PC into the SC. The most striking result was the observation in the intercellular lipid spaces of vesicular structures (curved arrow) of about 180 nm. (micrographs B and C). Given that these structures were not observed neither in the native SC (Fig. 2) or in the tissue treated with OG without liposome incubation (Fig 3), and taking into account their similarity in size and topology with PC vesicles (Fig 1), we could associate these structures to PC vesicles. However, bearing in mind the size of PC vesicles (180 nm) and the interlamellar distance of the ordered lipids (about 5-8 nm) the direct penetration of these vesicles into the SC appears to be difficult.

In order to shed light on the mechanism of incorporation of PC vesicles inside the intercellular region of SC we determined the amounts of PC and OG incorporated into the SC during the two consecutive treatments using the TLC/FID technique. The total weight of PC and OG incorporated in 15 mg of tissue were 2.04 and 6.84 μ moles respectively, being the corresponding OG/PC molar ratio (Ra) of 3.35. This molar ratio corresponded to a system mainly formed by OG/PC mixed micelles [16].

In a previous work Olsson et al. [17] reported the formation of liposomes by continuous dilution of OG/PC mixed micellar solutions. We therefore hypothesize that the presence of PC liposomes inside the SC was due to the reconstitution of these vesicles in intercellular lipid spaces by dilution of the OG/PC micellar aggregates incorporated into the SC. In order to demonstrate this hypothesis a series of DLS experiments was performed to know the variation of the mean hydrodynamic diameter (HD) of the micellar solution of OG/PC (Ra 3.35) during its continuous dilution with buffer. The curve obtained is plotted in Figure 5.



It can be seen that the HD of particles increased up to a maximum was achieved (from 9 to 190 nm), indicating the reconstitution of the vesicles by dilution of the mixed micelles. From these findings we assume that the presence of PC vesicles in the intercellular lipid region of the SC observed by HRLTEM (Fig. 4, micrographs B and C) is the result of the reconstitution of these structures inside the SC by means of a three steps process: First, formation in the SC surface of a OG/PC micellar system between the PC adsorbed during the incubation with liposomes and the OG added in the subsequent treatment. Second, incorporation of this micellar system into the intercellular lipid region during the surfactant treatment (incubation of 18 h after sonication). The fact that no PC was found in the treatment bath corroborates this process of incorporation. Third, reconstitution of PC vesicles inside the lipid spaces by dilution of this micellar system during the final washing of the SC. After reconstitution, the big size of the vesicles formed hinder their migration outside the tissue through the intercellular lipid spaces (distance of the ordered lipids of about 5-8 nm) by simple washing. This new strategy that favours the incorporation of PC vesicles inside the OG gives light on the controversy existing on the ability of PC liposomes to penetrate into this tissue and open up new avenues in the therapeutical applications of liposomes in skin.

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